Chapter 3

Retinoid X Receptor Signalling in the Specification of Skeletal Muscle Lineage

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1. Introduction

Pluripotent stem cells have the capacity to develop into different cell lineages, and can be promoted into skeletal muscle lineage through the use of small molecule inducers. Retinoic acid (RA) signaling through the retinoic acid receptor (RAR) and retinoid X receptor (RXR), is important for embryonic development, and is able to enhance myogenic differentiation *in vitro* if used in combination with other small molecule inducers. Nevertheless, it only yields moderate results in promoting the differentiation of embryonic stem (ES) cells into skeletal myocytes. RXR is also known to be essential for embryonic development, but it is generally considered to act as a silent partner for other nuclear receptors such as RAR. We recently discovered that RXR selective ligand efficiently induces myogenic differentiation in mouse ES cells which respond poorly to RA. In addition, myogenic differentiation, enhanced by the RXR ligand, is mediated through a RAR independent mechanism, and recapitulates closely the sequential events observed *in vivo.* Since ES cell differentiation represents the properties of early developing embryo, efficiently generating skeletal myocytes with RXR selective ligand provides means to further scrutinize signaling pathways in skeletal myogenesis, in view of developing cell-based therapies for skeletal muscle-related diseases. In this chapter, we attempt to provide an in-depth analysis of recent research findings and the current stage of knowledge in the field of skeletal myogenesis.

2. The retinoid X and retinoic acid receptors

RXR belongs to the nuclear hormone receptor superfamily, such as steroid hormone, thyroid hormone, vitamin D receptors, and nuclear receptors including RAR, PPAR, LXR and PXR (Szanto *et al.*, 2004). It is a very unique protein with the ability to form heterodimers with one third of the 48 other nuclear receptors (Mangelsdorf *et al.*, 1995) giving it the potential to converge a large array of signaling pathways. The RXR can form homodimers, permissive

heterodimers, and non-permissive heterodimers in a ligand-dependent or -independent manner (Tanaka and De Luca, 2009). When RXR forms homodimers or permissive heterodimers (with PPAR, LXR, PXR etc.), it is amenable to RXR ligand-dependant activation since the activation domain of the partner receptor is placed in proximity to RXR helixes. Once RXR is activated by the ligand, conformational changes cause direct stabilization of the activation domain of its partner (Gampe, Jr. *et al.*, 2000b). When RXR forms non-permissive heterodimers (with RAR, VDR, TR etc.), it is not activated by ligand, as the binding of the partner receptor to RXR allosterically inhibits it (Kurokawa *et al.*, 1994; Tanaka *et al.*, 2009). Furthermore, the activation domain of the partner is not located in proximity to ligand activated residues in the RXR interface (Bourguet *et al.*, 2000; Gampe, Jr. *et al.*, 2000b).

2.1. DNA binding

The receptor dimers of RXR and its partner, constitutively bind to specific DNA response elements in the promoters or enhancers of the genes they govern. DNA binding specificity is determined by the number of spacer nucleotides present between two direct repeats of the canonical binding sequence 5′-PuGGTCA (Leid *et al.*, 1992; Umesono and Evans, 1989). The RXR/RAR heterodimers bind to the retinoic acid response element (RARE) with a consensus half site separated by 2 or 5 nucleotides (DR2 or DR5), whereas the RXR homodimers bind to the retinoid X response element (RXRE) separated by only one nucleotide (DR1) (Tanaka *et al.*, 2009) (Figure 1). Selective response element recognition is due to a short sequence (the P box) located at the C-terminal base of the N-terminal C1 finger of the DNA binding domain (DBD) which interacts with the binding motif, and also due to a weak dimerization function which encompasses the N-terminal base of the CII finger (D-box) of the DBD (Danielsen *et al.*, 1989; Green *et al.*, 1988; Kumar and Chambon, 1988; Luisi *et al.*, 1991; Mader *et al.*, 1989; Umesono *et al.*, 1989). While RXR/RAR heterodimers bind more effectively to the RAREs than RXR homodimers, RXRs homodimers can bind RXREs with high affinity (Zhang *et al.*, 1992). RAREs can overlap with RXREs, and since RXR/RAR heterodimers bind with a higher affinity than RXR homodimers, (Tanaka *et al.*, 2009), this may interfere with RXR signaling.

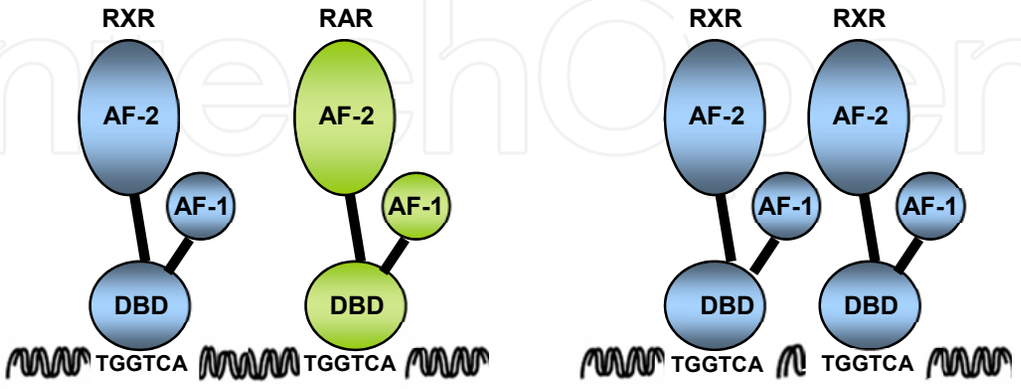

Figure 1. The Binding of RXR/RAR Heterodimer and RXR Homodimer to DNA. RXR/RAR heterodimers (left) and RXR homodimers (right) bind via the DNA binding domain to two direct repeats of the canonical binding sequence 5′-PuGGTCA separated by 2 or 5 nucleotides, or 1 nucleotide respectively.

2.2. Ligands of RXR and RAR

While RXR and RAR constitutively bind to DNA, they require agonist binding to activate gene transcription. Several endogenous ligands are well characterized and many synthetic ligands have been developed.

RA, the active derivative of vitamin A, can exist as two isoforms: all-trans RA and 9-cis RA. RAR bind and are activated by all-trans RA as well as its 9-cis isomer, while the RXR bind and are activated only by 9-cis RA (Ricaud *et al.*, 2005). However, due to the considerable difficulty of detecting 9-cis RA endogenously in embryos or in adult tissue (Niederreither and Dolle, 2008), there has been debate about the *in vivo* role of activated RXR, and has led to the belief that RXR serves only to orient and position the heterodimers properly on the DNA (Perlmann and Jansson, 1995; Willy *et al.*, 1995; Willy and Mangelsdorf, 1997)

In the last two decades, a wide range of RXR selective compounds has been engineered. The synthetic RXR ligands can act as agonists and activate both homodimers and permissive heterodimers. Conversely, they can also act antagonistically of homodimers, as is the case for the synthetic ligand LG100754, and promote only the activation of non-permissive heterodimers (Lala *et al.*, 1996). Bexarotene (LGD1069) is a synthetic RXR selective compound used in the treatment of cancer. It is unable to transactivate the RXR-RAR heterodimer (Lehmann *et al.*, 1992) and will not activate RARs (Nau *et al.*, 1999).

There are conflicting interpretations of RXR participation in the activation of RXR/RAR heterodimers. Some studies demonstrate that allosteric inhibition of RXR in the RXR/RAR heterodimer only occurs when the RAR is unliganded and that this inhibition is relieved once RAR is liganded (Forman *et al.*, 1995; Lala *et al.*, 1996). Other reports indicate that both receptors bind their ligands independently and that their effects are additive (Kersten *et al.*, 1995). The discrepancy between varying reports can possibly be reconciled by the fact that different ligands interact with distinct side chains in the ligand binding domain and thus mediate differential activation of the receptor complex. The exact response is therefore highly dependent on the identity of the ligand and cannot simply be classified as agonistic versus antagonistic. Although RXR can engage in ligand binding when RAR is ligand occupied and/or if a suitable synthetic ligand is present (Chen *et al.*, 1996; Kersten *et al.*, 1996; Lala *et al.*, 1996; Minucci *et al.*, 1997; Roy *et al.*, 1995), bexarotene is unable to transactivate the RXR/RAR heterodimer (Lehmann *et al.*, 1992). In fact, bexarotene has been reported to reduce interactions between RXRs and RARs whereas ligand such as 9-cis increases the binding of RXRs to RARs (Dong and Noy, 1998).

All-trans RA does not bind RXR (Mangelsdorf *et al.*, 1992), and more importantly, although all-trans RA has the ability to isomerize to 9-cis RA, pharmacological doses of all-trans RA are required to generate enough 9-cis to activate the RXRs (Mic *et al.*, 2003). Optimal enhancement of skeletal myogenic differentiation requires low concentrations of all-trans RA. Thus, all-trans RA isomerization is simply not a feasible explanation to the similar enhancement of myogenic differentiation by RA and bexarotene observed in P19 stem cells

(Le May *et al.*, 2011). Finally, while RA metabolites, such as 4-oxo-RA, were originally believed to play a role in RA signaling, they have more recently been shown as physiologically not required (Niederreither *et al.*, 2002; Pijnappel *et al.*, 1993).

2.3. The interaction of RXR and RAR with their cofactors

In response to ligand activation, RXR and RAR bind co-activators and the respective binding of cofactors again depends on the identity of the ligand. Agonist binding induces large conformational changes within the receptor causing helix 11 and 12 (the AF-2 domain) to close the lid of the ligand binding pocket and generate high affinity co-activator binding sites. This charged surface has a high affinity for a specific amino acid motif, LXXLL, which mediates the binding of co-activators to nuclear receptors (Westin *et al.*, 1998). Alternatively, if an antagonist or partial agonist binds, helix 12 is repositioned to an adjacent groove on the LBD and a charged surface that favors the co-repressor binding motif is formed (Perissi *et al.*, 1999).

Co-activators, as their name implies, have the ability to activate transcription and interact with the basal transcriptional machinery, bridge and direct the assembly of transcriptional pre-initiation complexes, and induce chromatin remodeling (Rosenfeld *et al.*, 2006; Bastien and Rochette-Egly, 2004). Co-activators such as p300, CREB Binding Protein (CBP), and p300/CBP-Associated Factor (PCAF) can all act as histone acetyltransferases (HATs) (Niederreither *et al.*, 2008; Ogryzko *et al.*, 1996) and form large multimolecular complexes.

Interestingly, co-activators p300 and CBP are also able to acetylate proteins other than histones, such as transcription factors (Gu and Roeder, 1997; Li *et al*. 1998; Li *et al*. 1999). CBP and p300 are heavily autoacetylated and upon recruitment to the receptors, can acetylate more of themselves in an intermolecular fashion (Karanam *et al.*, 2006). In addition to this, they have the ability to recruit PCAF (Yang *et al.*, 1996), a coactivator involved in myogenesis. p300 influences RXR activity as RXR are subjects for p300 acetylation, which promotes their binding to RXRE and increases their transcriptional activity as well (Zhao *et al.*, 2007). Co-activators play crucial roles in gene activation, however, those recruited by particular RXR dimers at specific genetic loci in response to ligand have yet to be identified.

Alternatively, in the absence of ligand, the co-repressors, such as the nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) family, bind and recruit a mulitprotein complex containing the histone deacetylase HDAC3 (Guenther *et al.*, 2000; Li *et al.*, 2000). The recruited histone methyl-transferases and histone deacetylases stabilize the nucleosome structure so that the DNA is inaccessible for transcription (Niederreither *et al.*, 2008).

When RXR forms permissive heterodimers (i.e.: RXR/PPAR), neither receptor binds the co-repressors under normal circumstances (DiRenzo *et al.*, 1997). Ligand binding to one receptor recruits the co-activators and although the other receptor may be unliganded, the high local concentration of bound co-activators favor the docking of the second LXXLL motif with the co-activator binding sites of the other receptor. If ligand is present for both

receptors of the permissive heterodimer, they can synergistically recruit co-activators (Ahuja *et al.*, 2003).

Non-permissive heterodimers (i.e.: RXR/RAR) do bind co-repressors and this binding to unliganded RXR and its partner is stabilized by both receptors. Transactivation requires ligand binding to the RXR partner (i.e.: RAR) to convert it into the agonist conformation, displace co-repressors, and recruit co-activators (Zhang *et al.*, 1999; Vivat *et al.*, 1997). As with permissive heterodimers, synergistic recruitment of co-activators occurs when ligands are present for both receptors (Ahuja *et al.*, 2003).

2.4. RXR and RAR in development

Gene mutation studies have determined that both RXR and RAR are essential for proper development, and delineated roles and tissue expression patterns for the different isoforms of the two receptors (α, β, and γ). The different RAR and RXR isotypes are encoded by different genes and their isoforms differ in their NH2-terminal regions which are generated by differential promoter usage and alternative splicing (Chiba *et al.*, 1997). While RXR-α null embryos show defects in RXR/PPARγ (Peroxisome Proliferator Activated Receptor) signaling, the RARs appear to be the most important partners for RXRs (Ahuja *et al.*, 2003). RXR/RAR non-permissive heterodimers have been extensively studied in the context of development.

During development, RXR-α and β are ubiquitously expressed with the highest levels of RXR-α present in the liver, heart, intestines, kidney, spleen, placenta, and the epidermis (Ahuja *et al.*, 2003; Pratt *et al.*, 1998). RXR-γ is expressed in all developing skeletal and cardiac muscles, the anterior pituitary, and the brain. The expression of RXR isoforms is tissue specific and often overlaps, yet occasionally certain isoforms are uniquely expressed.(Mangelsdorf *et al.*, 1992) RXR-α is the primary isoform and supports the activity of all three RARs. Furthermore, RXR-α may be important in the expression of RXRγ since the RXRγ gene contains a RXRE (Barger and Kelly, 1997).

Studies with mice lacking expression of RXR-α have found that these mice die in utero as a result of hypoplastic myocardium (Kastner *et al.*, 1994; Sucov *et al.*, 1994) and RXR-α null mutations exhibit growth retardation, webbed digits (Mark *et al.*, 2006) and defects in the chorioallantoic placenta (Sapin *et al.*, 1997). Loss of RXR-β and RXR-γ is not as severe since they can be compensated for by RXR-α (Tanaka *et al.*, 2009), which may explain why the RXR$\gamma^{-/-}$ mouse mutants are viable and have no muscular defects even in compound mutant combinations (Dolle, 2009).

Similarly, animals lacking RAR-α or RAR-γ result in postpartum lethality (Lohnes *et al.*, 1993). In RAR knock-out studies where two RARs are deleted, the mutants display a spectrum of defects that resemble vitamin A deficiency syndrome (Lohnes *et al.*, 1993; Lufkin *et al.*, 1993) and the function of the residual RAR is highly dependent on RXR-α (Ahuja *et al.*, 2003).

Even in normal development, the RARs are highly dependent on the RXRs. Homodimer formation of RARs is energetically unfavored, because of the limited contact between the interfaces. Pairing with RXR creates an extended area of intermolecular contact that stabilizes the heterodimer formation. This substantially larger surface area and consequent stability, results in the preferential formation of RXR/RAR heterodimers (Bourguet *et al.*, 2000; Gampe, Jr. *et al.*, 2000a).

3. Skeletal myogenesis

Skeletal muscle forms in the embryo from paraxial mesoderm, which segments into somites on either side of the neural tube and notochord (Christ and Ordahl, 1995). Extracellular signals from surrounding tissues play a significant role in muscle development. These signals include members of the Wnt family, specifically Wnt1 and Wnt7a secreted from the neural tube and surface ectoderm (Cossu and Borello, 1999), Sonic Hedge Hog (Shh) secreted by notochord and floor plate cells and which acts in conjunction with Wnt1 (Cossu *et al.*, 1999), bone morphogenetic protein4 (BMP4) secreted by the lateral plate mesoderm cells (Borycki *et al.*, 1999; Dietrich *et al.*, 1998; Munsterberg *et al.*, 1995; Pourquie *et al.*, 1996; Tajbakhsh *et al.*, 1998), and RA which is under tight regulatory control for its synthesis, degradation, and transport (Rohwedel *et al.*, 1999). These act on downstream targets such as HOX genes, which controls specification of the body axis (Rohwedel *et al.*, 1999), Brachyury T, a protein required for posterior mesoderm and notochord differentiation (Skerjanc, 1999), and the myogenic regulatory factors (MRFs) including Myf5, MyoD, myogenin, and Mrf4 which are required for the commitment and maturation of skeletal muscle (Cossu *et al.*, 1999; Rohwedel *et al.*, 1999; Skerjanc, 1999).

3.1. Myogenic regulatory factors and their cofactors

The formation of myoblasts from myogenic progenitors and their successive cell cycle arrest and differentiation into mature skeletal muscle involves two key families of transcription factors. The MyoD family of basic Helix-Loop-Helix (bHLH) proteins which includes the four master transcriptional regulators (also referred to as MRFs): Myf5, MyoD, myogenin, and Mrf4 (Arnold and Braun, 2000; Braun *et al.*, 1989; Braun *et al.*, 1990; Davis *et al.*, 1987; Edmondson and Olson, 1990; Froeschle *et al.*, 1998) and the myocyte enhancer factor 2 (MEF2) family of MADS-box transcription factors which includes MEF2A, -B, -C, and –D (Naya and Olson, 1999).

Myf5 and MyoD are involved in skeletal muscle specification and commitment and have the capacity of remodeling chromatin and opening gene loci that participate in further muscle differentiation (Bergstrom and Tapscott, 2001), whereas terminal differentiation is governed by myogenin and MRF4. Each MRF is sufficient to dominantly induce myogenesis when introduced into a variety of non-muscle cells (Olson, 1990; Weintraub, 1993), and ectopic expression of MyoD can inhibit cell cycle before the S phase independently of its DNA

binding and the induction of myogenic differentiation (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990).

Members of the MEF2 family alone are not sufficient to induce myogenesis, however the ability of the MRFs to convert cells is reliant on the function of the MEF2 family. MEF2 proteins bind as homodimers and heterodimers to the consensus sequence YTA(A/T)4TAR found in the promoter region of nearly every known muscle-specific gene (Black and Olson, 1998), and together with the myogenic bHLH proteins, synergistically activate the transcription of myogenic genes. Unlike the MRFs, MEF2 genes are also expressed outside the skeletal muscle lineage in tissues such as cardiac and smooth muscle (Black *et al.*, 1998; Edmondson *et al.*, 1994; Leifer *et al.*, 1993; Lyons *et al.*, 1995).

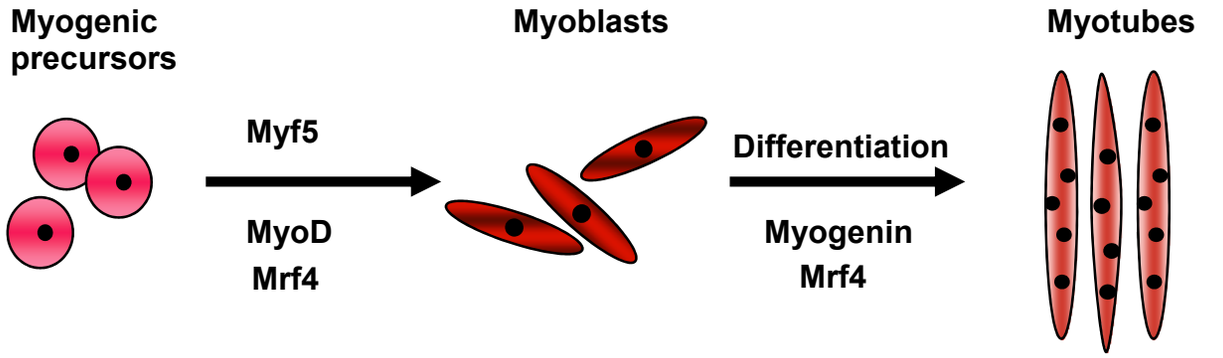

Figure 2. Involvement of Myogenic Regulatory Factors in Myogenesis. Myf5 and MyoD are involved in specification and commitment of muscle progenitors into skeletal muscle lineage. Mrf4 also plays a role as a determination gene in addition to directing terminal differentiation along with myogenin.

The bHLH domain of the MRFs is responsible for DNA binding and for dimerization with the ubiquitously expressed bHLH E protein (Hu *et al.*, 1992; Murre *et al.*, 1989; Parker *et al.*, 2006). The resulting myogenic bHLH-E heterodimers bind to DNA at the consensus sequences known as an E-box (CANNTG), specific DNA motifs present at muscle gene enhancers and/or promoters, where they regulate gene expression (Sartorelli and Caretti, 2005). These genes include cytoskeletal, sarcomeric, metabolic, and cell signaling proteins (Angus *et al.*, 2001; Gramolini and Jasmin, 1999; Kraner *et al.*, 1999; Li and Capetanaki, 1993; Lin *et al.*, 1991; Marsh *et al.*, 1998; Shield *et al.*, 1996; Simon and Burden, 1993; Wheeler *et al.*, 1999). A requirement for the MyoD family of transcription factors in this combinatorial complex is demonstrated by the fact that the E protein homodimers bind to the same DNA sequences as the MyoD-E protein heterodimers, yet only the MyoD-E protein complex can cooperate with MEF2 factors (Naya *et al.*, 1999). Furthermore, the MRFs and MEF2 factors activate and repress each others transcription in a complex network (Arnold and Winter, 1998; Bergstrom *et al.*, 2002; Cserjesi and Olson, 1991; Olson and Klein, 1994; Wong *et al.*, 1994). For example, expression of myogenin requires MEF2, while myogenin activates the expression of MEF2 independently of other skeletal gene products (Cserjesi *et al.*, 1991; Ridgeway *et al.*, 2000). Similarly, MRFs can positively regulate their own transcription and

transcription of each other, creating positive auto- and cross-regulatory loops (Braun *et al.*, 1989; Thayer *et al.*, 1989)

3.2. Roles of meox and pax in the specification of myogenic progenitors

Signals from surrounding tissues activate the premyogenic program, and result in the expression of transcription factors such as Pax3, and Meox1/2 that specify cells into the skeletal muscle lineage and mediate the induction of the MRFs (McDermott *et al.*, 2005; Petropoulos and Skerjanc, 2002; Petropoulos *et al.*, 2004; Ridgeway and Skerjanc, 2001; Williams and Ordahl, 1994).

Pax3, a transcription factor with homeo and paired domain motifs, is thought to be activated by Wnt6a (Fan *et al.*, 1997) and is responsible for both delamination and migration of muscle progenitors to the limb bud (Tajbakhsh *et al.*, 1997). Pax3 is initially expressed throughout the somite before becoming restricted to the dermomyotome and subsequently the migratory muscle progenitor cells (Goulding *et al.*, 1994; Williams *et al.*, 1994). The importance of Pax3 in the delamination and migration of muscle progenitor cells is highlighted by the fact that mice which are Pax3 null have severe muscle loss (Alvares *et al.*, 2003; Bladt *et al.*, 1995; Dietrich *et al.*, 1999; Epstein *et al.*, 1996; Grifone *et al.*, 2005).

Pax3 directly regulates the expression of Myf5 through the limb bud enhancer of Myf5 gene (Bajard *et al.*, 2006) and acts with Myf5, upstream of MyoD which cannot be properly expressed in the Pax3/Myf5 double knockout (Tajbakhsh *et al.*, 1997). It is when the migrating cells reach the limb bud that they begin to express Myf5 and MyoD, and it is both before and after activation of these genes that the cells undergo extensive proliferation (Buckingham *et al.*, 2003; Tajbakhsh and Buckingham, 1994). Pax3, along with additional factors such as Myf5, c-met, Msx1 and the fibroblast growth factor (FGF) family of receptors promote myoblast proliferation. Proliferation is arrested by inhibitory signals which promote differentiation by inducing cell cycle arrest proteins such as MyoD (Alric *et al.*, 1998).

Meox1 and Meox2 are closely related homeobox genes with mesoderm and mesenchyme specific expression during mouse development (Candia *et al.*, 1992). Meox1 is expressed in the dermomyotome whereas after delamination and migration to the limb bud, Meox2 is predominantly expressed (Candia *et al.*, 1992; Candia and Wright, 1996). In Meox2 deficient limb buds, Pax3 and Myf5 are downregulated and mice homozygous for a null mutation in Meox2 have defects in limb muscle differentiation resulting in an overall reduction in muscle mass and absence of specific muscles (Mankoo *et al.*, 1999). It is only the compound mutant embryos of $Meox1^{-/-}$; $Meox2^{-/-}$ that display a dramatic phenotype associated with disrupted somite development. In these embryos, the axial skeleton fails to develop and most skeletal muscles are absent or reduced in size (Mankoo *et al.*, 2003). Interestingly, in cell cultures, overexpression of Meox1 does not induce myogenesis and while a dominant negative Meox1 has been shown to downregulate Pax3 and Gli2 expression and inhibit myogenesis in the P19 stem cells (Petropoulos *et al.*, 2004), Meox1 mutant mice exhibit mild defects in sclerotome-derived vertebral and rib bones (Mankoo *et al.*, 2003) rather than showing any overt muscle defects.

3.3. Roles of histone acetyltransferases in myogenic expression

Not only are extracellular signals crucial for the proper induction of MRFs, but intracellular prompts involving acetyletransferases play a fundamental role as well. CBP and p300 are required for growth arrest and apoptosis (Vo and Goodman, 2001), and along with PCAF are required for terminal differentiation of myoblasts and transactivation of muscle specific promoters such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) (Eckner *et al.*, 1996; Polesskaya *et al.*, 2001; Puri *et al.*, 1997a; Puri *et al.*, 1997b; Yuan *et al.*, 1996). Embryonic stem cells lacking p300 or its HAT activity are strongly impaired in their ability to activate Myf5 and MyoD (Roth *et al.*, 2003). When properly expressed, Myf5 and MyoD, in cooperation with MEF2 transcription factors and with p300 and CBP, mediate the activation of the secondary MRFs, myogenin and Mrf4. p300 has been shown to bind directly to MyoD (Sartorelli *et al.*, 1997; Yuan *et al.*, 1996), and both p300 and PCAF play a critical role in the maximal MyoD dependant transactivation; p300 acetylates histones H3 and H4 and recruits PCAF to the promoter whereas PCAF acetylates MyoD to enhance transcription initiation, increase its affinity for DNA binding, and facilitate heterodimer formation with E proteins (Dilworth *et al.*, 2004; Puri *et al.*, 1997a; Sartorelli *et al.*, 1999). However, MyoD has also been found to be acetylated in proliferating myoblasts where it is inactive, therefore further mechanisms besides simply acetylation are required for MyoD activation (Polesskaya *et al.*, 2000).

4. Impact of extracellular cues on MRF expression

Ligands of RAR and RXR play important roles in the activation of myogenesis and this activation is highly dependent on the identity of the ligand. RA is required for proper somite formation (Maden *et al.*, 1996; Maden *et al.*, 2000; Niederreither *et al.*, 1999), induction of specification genes Meox1, Meox2, and Pax3, and counteracts inhibitory signals such as BMP4 (Kennedy *et al.*, 2009). RA signaling intersects with that of BMP4, as BMP4 and RA function antagonistically and have the capacity to counteract each other's inhibition of entry into skeletal and cardiac muscle lineages (Kennedy *et al.*, 2009). Low concentrations of RA can regulate the levels of Myf5 implying the existence of a RARE in the Myf5 regulatory region (Carnac *et al.*, 1993). RA also enhances MyoD and myogenin expression (Carnac *et al.*, 1993), and RA receptors and MyoD have been found to upregulate each other's transcriptional activity; their transcriptional co-activation requires a RA receptor-MyoD complex that binds to MyoD DNA binding sites in muscle cells (Froeschle *et al.*, 1998). RA is capable of inhibiting proliferation of myoblasts through inducing cell cycle arrest proteins (Alric *et al.*, 1998) and in vitamin A deficient embryos, myogenin is downregulated (Maden *et al.*, 2000) providing a link between RA and myoblast maturation.

RA and bexarotene are both capable of inducing skeletal myogenesis in the P19 stem cells, however, they do so through differential activation of crucial specification genes (Le May *et al.*, 2011). Bexarotene primarily activates Meox1 while RA mainly activates Pax3. Nontheless, both ligands are equally capable of inducing later target genes such as MyoD and myogenin. Alternatively, only bexarotene is capable of inducing myogenesis in ES cells

to a significant level (Le May *et al.*, 2011). Furthermore, treatment of these cells with bexarotene gives long, mature, multinucleated myofibers.

4.1. Stem cell as a model for study of myogenic differentiation

It is highly advantageous to use stem cell tissue cultures to study the importance of specification genes in a controlled environment to understand their relationship with each other and their regulation by extracellular signaling molecules. Specification factors exist in a very complex relationship and have the ability to autoregulate and cross-regulate one another (Petropoulos *et al.*, 2004).

In P19 stem cell cultures, Pax3 overexpression can induce Meox1 but is unable to activate Gli2 and a dominant negative Pax3 mutation does not affect Gli2 levels. Conversely, Gli2, which also has the ability to upregulate Meox1, can upregulate Pax3 while the dominant negative Gli2 P19 cells downregulate Meox1, Pax3, and MyoD expression and inhibits myogenesis. Lastly, Meox1 can activate the expression of Gli2 but overexpression of this protein is insufficient to induce Pax3 or skeletal myogenesis (Petropoulos *et al.*, 2004). The ability of each of these factors to induce each other, or, in their absence, completely abolish myogenesis underlines the importance of these factors in the specification process.

Wnt signaling via β-catenin is also essential and sufficient for the induction of specification factors Pax3, Meox1, and Gli2 and in P19 stem cells, a dominant negative β-catenin inhibits Pax3, Gli2, Meox1 and MyoD expression and abolishes myogenesis (Petropoulos *et al.*, 2002). This is not surprising since mutations of either Gli2, Meox1, or Pax3 in these cells will abrogate myogenesis (Petropoulos *et al.*, 2004). Pax3 expression is essential and sufficient for the expression of the transcription factor Six1 and the induction of skeletal myogenesis (Ridgeway *et al.*, 2001). Its overexpression induces Myf-5, MyoD, and myogenin expression (Maroto *et al.*, 1997) whereas a dominant negative Pax3 in P19 cells results in a loss of MyoD and myogenin expression and subsequent myogenesis (Ridgeway *et al.*, 2001).

4.2. Significance of a separate RXR signaling pathway

The importance of a separate, rexinoid signaling pathway in skeletal muscle development non-overlapping with RA signal transduction is demonstrated by the fact that an RXR selective ligand, bexarotene effectively enhances skeletal myogenesis in mouse ES cells that respond poorly to RA (Le May *et al.*, 2011). This difference in the two signaling pathways stems from differential activation of very early genes involved in crucial lineage specification, although both bexarotene and RA are dependent on functional β-catenin signaling (Le May *et al.*, 2011). It is intriguing that a cell type such as ES cells, that has thus far been relatively resistant to RA-induced skeletal muscle differentiation develops so well in the presence of an RXR selective ligand, especially considering these cells do not posse the necessary machinery to synthesize 9-cis RA, the purported endogenous ligand (Chen and Khillan, 2010). It appears that P19 cells have the ability to differentiate by both retinoid and rexinoid signaling instigated pathways while ES cells respond well only to rexinoid

mediated pathways. Similarly, RAC65 cells resistant to RA-induced skeletal muscle and neuronal conversion (Costa and McBurney, 1996) demonstrate efficient skeletal differentiation when treated with RXR selective ligand (Le May *et al.*, 2011). The ability of rexinoid to bypass the dominant negative RAR inhibition in RAC65 cells is not unique to skeletal muscle and has also been documented for neuronal differentiation as well (Yokota and Ohkubo, 1996). Finally, RXR is able to activate target genes involved in RA signaling that cannot be induced by RARs as is the case with the response element in the CRBPII (Cellular Retinol Binding Protein Type II) gene which contains a DR1, underscoring the possibility of RXR/RXR and RXR/RAR independent pathways (Mangelsdorf *et al.*, 1991).

It remains to be determined which specific co-activators are recruited by RXR in the enhancers or promoters of target genes during skeletal myogenesis. RXR homodimers or RXR permissive heterodimers might recruit a separate set of co-activators and therefore differentially control gene expression. It could be that the unique ability of bexarotene versus RA to control the transcription factor's interactions with co-activators is the method by which distinct and even competing signaling pathways can be distinguished.

4.3. Unsaturated fatty acids activate RXR

The physiological significance of 9-cis RA signaling is debated due to a lack of consensus on its existence in the developing embryo. However, the enzymes that contribute to its biosynthesis are well documented (Mertz *et al.*, 1997; Romert *et al.*, 1998) in addition to its ability to induce the formation of homodimers that bind to DR1 sequences (Zhang *et al.*, 1992). The lack of a known ligand is hardly reason to exclude RXR as physiologically significant *in vivo* and a major factor that supports the presence of an active endogenous ligand is the fact that RXR tetramers cannot dissociate without agonist binding.

Studies using RXR ligand-detector mice have identified specific regions of the spinal cord as major sites of endogenous rexinoid production and classify naturally occurring poly-unsaturated fatty acids, including docosahexaenoic acid (DHA) as a major endogenous ligand for RXR in the mouse brain (Ahuja *et al.*, 2003; de Urquiza *et al.*, 2000). When characterized in the ligand binding domain of RXR-α, DHA has a significantly higher number of ligand-protein contacts than 9-cis and certain synthetic ligands and also has the ability to activate RXR homodimers as well as synergistically activate the RXR-RAR heterodimers in combination with all-trans RA (Lengqvist *et al.*, 2004). It remains to be determined if this ligand is functional in all tissues or whether there are other yet undiscovered ligands. Presently, additional unsaturated fatty acids, including docosapentaenoic, arachidonic, and oleic acids, also have been found to bind and activate RXR, suggesting that this ability is not exclusive for DHA. Irrespective of whether an endogenous RXR ligand does indeed exist, the ability to control cell growth and differentiation through targeting RXR with highly selective ligands confers *many* therapeutic applications to this unique receptor.

5. Therapeutic potentials of rexinoids

It is unknown whether RXR homodimer or RXR permissive heterodimer signaling is the main mechanism governing skeletal muscle differentiation. Regardless, controlling cell processes using RXR selective ligands underlines the fact that two distinct and possibly overlapping pathways exist. Moreover, RAR-independent rexinoid signaling provides another route of achieving cell cycle arrest and differentiation when RA signaling is aberrant, a situation frequently seen in cancer where differentiation often appears to result in loss of a malignant phenotype (Gokhale *et al.*, 2000).

RXR-α overexpression sensitizes tumors to rexinoid-induced anti-growth effects, cellular differentiation, decreased cell proliferation, apoptosis of some type of cancer cells, and prevention of angiogenesis and metastasis (Qu and Tang, 2010). Bexarotene, has been approved by the FDA for use in the treatment of refractory or persistent cutaneous T-cell lymphoma and has the ability to reduce tumor development in several other cancers (Duvic *et al.*, 2001; Wu *et al.*, 2002). However, the use of this compound in the treatment of lung and breast carcinomas has yielded disappointing results (Tanaka *et al.*, 2009) demonstrating our lack of understanding of the molecular mechanisms underlying rexinoid-induced antitumor effects and RXR-induced multi-pathway activation.

One of the reasons rexinoids seem such promising chemotherapeutic compounds compared to retinoids, is that retinoids have numerous side effects which severely limit the dosage and efficacy while rexinoids display mild toxicity. Furthermore, RXR expression is rarely lost in human tumors whereas RAR expression is frequently lost or reduced in various cancers (Sun and Lotan, 2002; Umesono *et al.*, 1989). Since p53 abnormalities are reported in more than 50% of human cancers, and p21 is rarely mutated (Shiohara *et al.*, 1994; Tanaka *et al.*, 2007), RXR mediated induction of p21 is a promising therapeutic target for these cancers. The study of myogenic differentiation may provide some answers to new target genes as the development and progression of cancer involves aberrations in the same mechanisms that regulate cell differentiation during embryogenesis. It remains to be revealed which other genes can also be targeted by rexinoids and which specific interactions take place that we can study and apply to our development of more potent and effective therapeutics.

Pluripotent stem cells closely simulate embryonic development and present a model system with which to dissect signaling pathways of target receptors in controlled environments. They hold a tremendous potential for cell-based therapies through their capacity to grow and regenerate new tissues. Many diseases including muscular dystrophies, cancer, AIDS, and even normal conditions such as aging show prominent muscle loss that would benefit enormously from regenerative cell-based therapies. However, our ability to use stem cells in muscle-wasting disorders has been limited due to the low rate of myogenic differentiation in ES cell cultures and the difficulty in identifying and isolating progenitor cells. To harvest the full potential of these cells in therapies, it is imperative that we find small molecule inducers capable of efficiently directing stem cells into skeletal muscle lineage. Attempts at using RA in ES cell cultures have thus far yielded disappointing results; however, the ability of

rexinoids to induce these cells has not yet been fully explored. Understanding the myogenic pathway *in vivo* as well as deciphering differentiation cues to culture pure populations of myogenic progenitors will prove a vital tool in the treatment of such devastating diseases.

6. Conclusion

RXR selective ligand is an effective inducer of skeletal myogenesis not only in the P19 pluripotent stem cells, but also in the mouse ES cells which have thus far been relatively resistant to RA induction. RXR specific signaling plays an important role in this process through a separate RAR-independent mediated pathway. It appears that RA and rexinoid enhance skeletal myogenesis through differential activation of early developmental genes. Our study demonstrates that activation of RXR causes an increase in the mesodermal Meox1 gene while RA induces the skeletal specific gene Pax3. It will be interesting to uncover other novel genes targeted by rexinoid. Determining the molecular mechanism by which rexinoid exerts its effects to enhance skeletal myogenesis is challenging due in part to the complexity of the developmental systems in which it exerts its effects as well as the intricate relationship of protein complexes and gene regulation. Since ES cells closely recapitulate the properties of the developing embryo, elucidating these molecular pathways will be imperative in the manipulation of stem cell progenitors and aid in the generation of pure populations of skeletal myocytes to use in the treatment of muscle-related diseases.

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